INVESTIGATING THE EFFECTS OF OPHRYOCYSTIS ELEKTROSCIRRHA ON THE MONARCH BUTTERFLY (DANAUS PLEXIPPUS)

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by

Christian A. Mandujano

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ABSTRACT

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Monarch butterflies of the genus *Danaus* are an iconic species that have three large populations spanning three geographic locations in the United States; the west coast of California, the east coast stretching over Florida to the Carolinas, and the Central-Midwest population that migrates every year. Over the past 30 years, some studies have indicated that the monarch population is dwindling due to several factors. These include many abiotic and biotic factors such as parasites, bacteria, viruses, logging in Michoacán (winter roost site), and climate change which are currently having detrimental effects. This study aims to investigate one of these detrimental factors, namely the parasitic infection of young instar larva by an intracellular neogregarine parasite. *Ophryocystis elektroscirrha* is an intracellular Apicomplexa parasite that infects the larval stages of monarch butterfly development. To assess the pathology that occurs to the larva during parasitic growth, an experimental design was applied that purposely infect larval instar stages 2-5 with varying intensity of *O. elektroscirrha* spores (2,000, 12,500, 25,000). Histological sections were made and analyzed for micronuclear schizonts where individual specimens were measured and recorded. In order to get a more comprehensive understanding of the pathology occurring, hemolymph smears were taken and immune cell types were counted and identified to gauge the immunological response. I found that despite high infection intensity at various stages there does not appear to be much of a pathological or immunological response from the host organism, with a noticeable exception in stage five larva.

KEY WORDS: Monarch, *Ophryocystis elektroscirrha*, Pathology, Hypodermis, Infection, Infected

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CHAPTER I

Introduction

The Midwest population of monarchs (*Danaus plexippus*) has been declining for 17 years (Rendon-Salinas et al., 2010). This species makes a 2-way migration to and from Mexico to North America every year (Brower et al. 2012). With the threat of anthropogenic climate change (IPCC., 2007), and its effects on the monarch population (Barve et al., 2012), coupled with destruction of forest habitats (Navarrete et al., 2011), it is not surprising that this species has been in decline. There are several hypotheses regarding the cause of decline in the midwest population ranging from changes in climate to the declining number of milkweed species and nectar plants that exist within the migratory pathway (Pleasants and Oberhauser, 2012; Inamine et al., 2016; Pleasants et al., 2017, Thogmartin et al., 2017; Marini et al., 2017).

The over-wintering populations of monarch butterflies face a long migration from their winter home in Michoacán, Mexico to their summer range that stretches from South Dakota to the eastern edge of the Great Lakes (Flockhart et al., 2013). Every year, this migration takes place to avoid the inhospitable environment which is characterized by low temperatures and decline of resources (i.e. milkweed, flowering plants), and to move to a warmer climate, where food and energy are abundant enough to sustain the population (S. M. Reppert and De Roode, 2018).

One of the dangers for migrating monarch populations is infection with the neogregarine *Ophryocystis elektroscirrha* (OE) (Apicomplexa:Neogregarinorida). These are obligate intracellular parasites that infect larval stages of monarch butterflies. Transmission occurs when the spore stage is dropped from the abdomen and carried on

the wings of infected monarchs. Two lepidopteran hosts are known to carry *Ophryocystis elektroscirrha* infections, the monarch butterfly (*Danaus plexippus*) and the queen butterfly (*Danaus gilippus*) (McLaughlin and Myers, 1970). *Ophryocystis elektroscirrha* depends on the movement and life cycle stages of monarch and queen butterflies to complete its life cycle.

The monarch's life cycle begins with an egg that is deposited on a milkweed leaf via the ovipositor of a female butterfly. Depending on the temperature, the larva remains in the egg for approximately 3-5 days. After emergence, the larva consumes the egg case, because it is close and nutrient rich. With optimal temperature and food availability, larval development accelerates over the next 30 days. Typically, there are 5 instar stages of development, each marked by an increase in body length and weight. Either by environmental or internal cues, the fifth instar larva begins to spin a silk mat and uses the last pair of prolegs to hang from the silk mat. As the larva begins to shed its exoskeleton, the cremaster, an anchorage point at the tip of the monarch's abdomen, is pushed into the silk pad, a multi-layered cushion of silk spinnings that are spit from the monarch larva's mouth. During this time in the chrysalis, developmental changes begin to occur. After approximately 10-15 days, a fully developed adult emerges and begins seeking resources and mates (Hunt and Tongen, 2016). It is during this entire cycle, from the laying of the egg on a milkweed leaf to the emergence of the adult monarch, that *Ophryocystis elektroscirrha* develops.

Ophryocystis elektroscirrha depends on its host for both survival and reproduction. During replication, the host is damaged by schizonts traveling through the gut wall and into the hypodermis. The schizonts then begin creating pockets in the

hypodermis where they reside, and pathology begins. Pathology includes decreased adult body size and shortened life span (De Roode 2007, 2008). Infected monarch populations can possibly evade further parasitism through the mechanisms of migratory culling, migratory escape, or a combination of the two. If the monarch butterflies can leave their contaminated habitats for resources farther away (migratory escape), or if those butterflies that are infected die prematurely and cannot make the long migratory journey (migratory culling), then it is thought that infection rates could decline (Bartel et al., 2011). However, when neither of these mechanisms occur, the problem is exaggerated, especially when taking into account that some *O. elektroscirrha* spores can survive harsh environmental conditions such as UV exposure, heat, and changing humidity (Satterfield et al., 2017). Moreover, the spores can still be infectious for 3-11 weeks, depending on the season (Satterfield et al., 2017). It is thought that *O. elektroscirrha* has a long-standing parasitic relationship with the butterfly population and may be contributing to the monarch population decline (Altizer et al., 2015). While studies have addressed the *O. elektroscirrha* life cycle and damage to host fitness (Altizer and Oberhauser, 1999), there remains a need to investigate tissue-level and cellular-level pathology.

The life cycle of *O. elektroscirrha* was first described by McLaughlin and Meyers (1970), where it was suggested that when a young monarch larva ingests milkweed leaves contaminated with *O. elektroscirrha* spores they become infected. The spores lyse when they have reached the host gut. The sporozoites penetrate the gut and travel to the hypodermis, where they begin to reproduce asexually in hypodermal cells (micronuclear schizogony). When the fifth larval instar begins metamorphosis, the parasite also changes and begins to reproduce sexually. While inside the hypodermal cells of the pupating

monarch, the schizonts, a single cell asexual stage, begin to metamorphose into merozoites, a sexually reproducing single cell stage. Macronuclear merozoites begin to round up into spherical gamonts to form a gametocyst where the nuclei begin to divide. Gametocysts begin to quickly form zygotes and replicate through the process of meiosis. During this time, the young pupating monarchs' hypodermal cells begin to metamorphose into the scales of an adult monarch butterfly. If the monarch lives through these steps, it will emerge with spores fully covering the outside of its body. Both developmental stages of *D. plexippus* and *O. elektroscirrha* are depicted side by side in Figure 1. The cycle will begin again once the adult female monarch begins to lay eggs and deposits spores onto the milkweed leaves (McLaughlin and Myers, 1970).

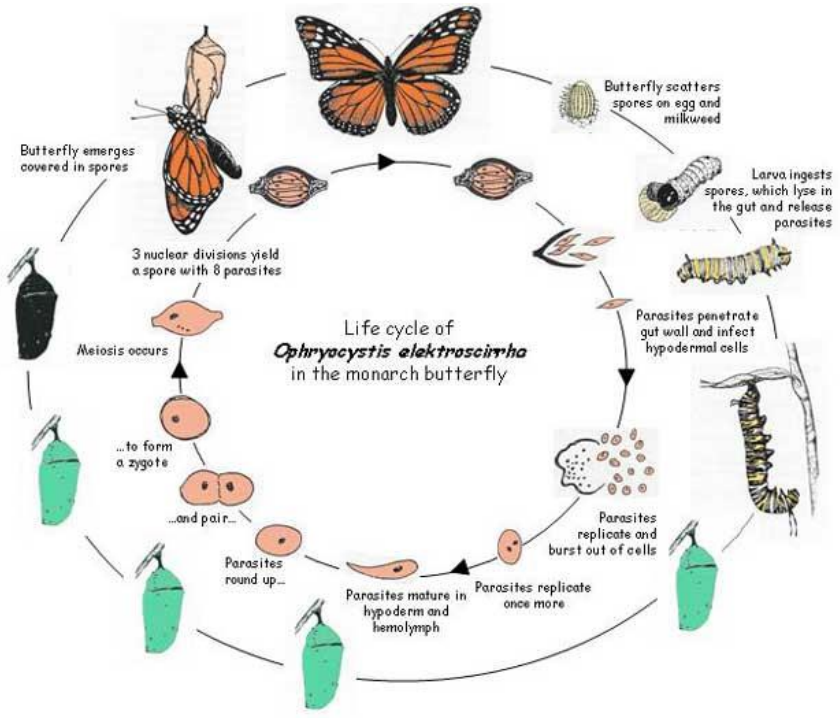

Figure 1. Detailed life cycle stages of both *Danaus plexippus* and *Ophryocystis elektroscirrha* (Copyright J.C. de Roode).

Currently, there are few studies addressing the pathology that *O. elektroscirrha* inflicts upon its host. Indirect consequences have been suggested, and a few studies have

addressed the fitness, longevity, and immune response but direct cellular or tissue level pathology have not been documented (Satterfield et al., 1999; De Roode et al., 2008; Lefevre et al., 2012; Satterfield et al., 2013). There have even been studies examining the relationship between monarchs, host plants (*Ascelpias*), and the parasite *O. elektroscirrha*. Tao et al., (2016) showed different concentrations and polarity of cardenolides from milkweed plants affect the survivability and infection levels of monarchs, and the results indicate that although resistance to *O. elektroscirrha* infection is conferred to the larva and adult stage, there is a trade-off. The larva have a decreased chance of survival from first instar to pupation when feeding on high foliar cardenolide milkweed, but infected adult monarchs have reduced parasite spore intensity when feeding on high foliar cardenolide milkweed. De Roode et al., (2008) demonstrated that adult monarchs had reduced spore intensity and longer life spans when reared on *Asclepias curassavica*, compared to those raised on different milkweed species, such as *Asclepias incarnata*, suggesting that host-plant relationships play an important role in determining *O. elektroscirrha* virulence. When tested with two other species of milkweed, *A. curassavica* and *A. incarnata*, the number of infectious spores that could initiate infection in their hosts seemed to be reduced. Both species of milkweed mitigated virulence when monarch larva were raised on a steady diet of the plant. This suggests that the preferred plant to rear monarch larva on is *A. curassavica*. (De Roode et al., 2011). The findings of both Tao et al., (2016) and De Roode et al., (2011) suggests a relationship between *O. elektroscirrha* spore intensity and the species of milkweed a monarch larva feeds. When a larva encounters several *O. elektroscirrha* spores, an *Asclepias* species with a high cardenolide concentration is preferred, as the cardenolides confer a better

resistance. In another study by Sternberg et al., (2015) the paternal heritage of the monarch larva seemed to pass down a resistance to its progeny, further bolstering support that rearing young monarch larva on *A. curassavica* is beneficial to survival and ensuring the monarch larva reach eclosion. Still, research is lacking on specific physical damage done to the monarch's hypodermal tissue. Similar studies have been conducted that examine histopathology of the cotton bollworm, *Helicoverpa armigera*, a lepidopteran infected with the polyhedrosis virus. Physical rupture of columnar cells in the epithelium along with a degeneration of the mitochondria and rough endoplasmic reticulum were found (Marzban et al., 2013). These findings have a tenuous link to the pathology formed by *O. elektroscirrha* but serve as a model when examining pathology found in lepidopterans. In a similar study examining various regions of lepidopterans infected by *Chelonus blackburni* a parasitoidal wasp, findings indicate that with the disruption of cell types such as columnar and goblet cells of the epithelium, nutritional deficiency would occur and lead to increased mortality rates (Sanap et al., 2016). It can be hypothesized that *O. elektroscirrha* infecting monarch hypodermal tissue causes similar damage, as well as robbing the larva of essential nutrients by utilizing those resources for reproduction. Because the majority of the damage in the early stages of the infection comes from physical destruction of the gut wall, as well as taking up physical space in the hypodermis, it can be hypothesized that most of the pathology found in *O. elektroscirrha* infections results from physical disruption of those tissues coupled with nutrient depletion. For this study, pathology was determined to be the amount of intracellular space taken up by the parasite in hypodermal tissue measured in μm. Pathology itself can manifest in several ways, from direct mechanical destruction of host

cells and tissue, release of certain chemical compounds that interrupt host cell function, to the host producing an immune cell response that can overwhelm the host causing disruption of normal bodily function and leading to death (Cabral and Cabral 2003, Haan and Hirst 2004, Cowman et al 2012). Of these several possibilities, any number of which could be the leading factor causing death amongst monarch larvae. Tissue area measured can be linked to pathological disruption and displacement by uptake of intracellular space by schizont infection, as has been seen and noted in similar modes of infection by intracellular parasites (Despommier 1974, Chatterjee et al 2000, Sotelo and Brutto 2002, Chimelli 2011). Mclaughlin and Myers (1970) demonstrated that *O. elektroscirrha* infecting the hypodermis resulted in host cell destruction; in this layer, there are epidermal cells that are nutrient rich because of the chitin that forms in the layers above. If *O. elektroscirrha* are infecting epidermal cells just below the chitinous layer, it can be postulated that in those cells, the micronuclear schizonts are causing most of the pathology. Other possible targets are cells such as the oenocytes (Figure 2) which are responsible for lipid production in the hypodermis (Martins and Ramalho-Ortigao, 2012). Because of the amount of available and easily accessible energy, the hypodermal layer and oenocytes are a possible target for *O. elektroscirrha* parasitism. Figure 2 details cells of the hypodermis that perform various tasks, such as secreting chitin across the apical membrane or allowing cuticular hairs or bristles to reach the surface of the cuticle. These cells are continuously supplied with oxygen and nutrients carried by the hemolymph, making the pathology of these cell types especially detrimental to host survival.

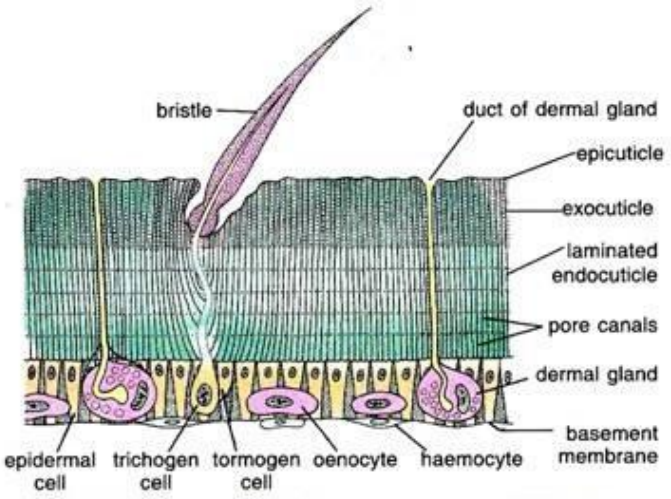

Figure 2. Detailed picture of basic insect cuticle, hair, and hypodermis with various cells (Copyright, (2013) Wiley. Used with permission from (Helmut Fritz van Emden, Chapter 2, Handbook of Agricultural Entomology, John Wiley and Sons).

As the fifth instar larva undergoes ecdysis, *O. elektroscirrha* continues its cycle and becomes embedded in the exocuticle and epicuticle, potentially disrupting the pattern of chitin microfibrils and pore canals that are important for sclerotization (McLaughlin and Meyer, 1970). Insects do not have an acquired immune system, but rather rely on innate systems which are subdivided into two major categories, humoral and cellular. Lepidopterans in particular have at least four differentiated hemocyte types: phagocytic granulocytes, capsule-forming plasmatocytes, spherule cells, and PO-containing oenocytenoids (Strand, 2008). Each of these cell types play critical roles in defending the host against pathogens, including bacteria, viruses, and parasites. Certain cell types, such as phagocytic granulocytes, encapsulate foreign bodies and begin enzymatic degradation if possible. If the pathogen is too large, then more specific cell types are introduced to contain the pathogen. Cell types, such as the PO-containing oenocytoids, will induce a

cascade which leads to the formation of proteins that are specifically synthesized to combat the pathogen (Tanaka et al., 2008).

It is also worth noting, however, that in some species, with the addition of a parasitic host, that certain functions that have been physically expanded beyond their expected capacity. This can have unintended and hidden consequences such as the distention and vacuolization of epithelial cells that occurs in Pacific oysters (Knowles et al., 2014). This may translate into the idea that with the expansion or distention of the hypodermal layer due to so many schizonts taking up residence, unforeseen damage may occur in those tissues or cells that are in close proximity to the infection site. Altizer and Oberhauser in their 1999 study, come closest to answering this question. They infected monarch larva with gradually increasing spore intensities. Their results demonstrated a decrease in sexual reproduction and host survival, suggesting that with a high intensity of parasites, internal pathology has drastic effects on monarch fitness.

Based upon findings of parasitism and infections in both monarch butterflies or similar lepidopteran species, a study was conducted that examined possible pathology to the hypoderm through histological examination. First, instar stages from 2-5 were infected with varying intensities of *O. elektroscirrha* spores (2,000, 12,500, 25,000), to determine if the number of schizonts found in hypodermal tissues would increase in number with the increase in spore intensity. The study also examined if the extent of pathology found in the tissues by schizonts taking up intracellular space increase with instar stage and or increasing inoculum intensities. The second question posed in the study asked if a visible disruption in the cuticular layer by *O. elektroscirrha* infection would manifest through increasing inoculum intensities and increasing stage of instar at

which inoculum was administered. The third question in the study looked at whether or not there would be an increase in the number of free-swimming parasite stages or parasitic spores found in the hemolymph and hypodermal tissue. Performing a statistical analysis on the total number of parasite stages found in tissue and hemolymph should be able to show if either variable being tested has an effect on parasite intensity. Finally, based upon current understanding of basic insect immunology, the study sought to determine if there would be an increased immune response in terms of the number of immune cells found on the hemolymph smears, and more specifically, an increase in certain immune cell types. An increase in total immune cell numbers could indicate an immunological response against foreign invaders in the hemolymph fluid of the instar larva. It is also important to examine changes in specific immune cell type, even if the overall total count is not grossly affected, this could indicate that certain parasite-immune cell relationships are occurring whereas with other immune cell types there are no direct or indirect interactions occurring.

CHAPTER II

Materials and Methods

Collection

Monarch butterflies were collected on the property of Sam Houston State University and two private residences within the city limits of Houston. The collection date, location, and sex of the specimen were recorded, and specimens were returned to the lab. A small piece of scotch tape was gently applied to the dorsal and ventral surfaces of each butterfly's abdomen, then placed on a microscope slide. Slides were examined at 100x magnification to determine whether or not the butterflies were infected with *O. elektroscirrha* spores. Entire adult butterflies were labeled and refrigerated to avoid potential desiccation of the spores.

Monarch eggs, donated from Houston Museum of Natural Science, Houston, TX, U.S.A., were washed in a five percent bleach solution and inspected for *O. elektroscirrha* spores to ensure no larvae were accidentally infected upon hatching. Each larva was reared in a separate petri dish to prevent cross contamination. Instars were reared and kept uninoculated until they reached the appropriate stage where they were then fed 5mm^2 *A. curassavica* leaf portions that had been inoculated with varying doses of *O. elektroscirrha* spores. *Ophryocystis elektroscirrha* spores were vortexed from the monarch abdomens and suspended in a Tween ™ solution. Spores were counted on 1X 10^{-4}ml grid-cells on four separate hemocytometer slides (Altizer and Oberhauser, 1999). Spores underwent a dilution series to 2,000, 12,500, and 25,000 spores/10µl. At each serial dilution, 30 larvae at each group (ex. Instar level 2, inoculum intensity 2,000) were inoculated by placing 10 µl aliquots of infected monarch solution on five mm^2 leaf

portions and an additional 30 larvae were inoculated with uninfected monarch solution. Larvae were raised until pre-pupae stage by keeping them isolated in their own petri dishes, and were continuously fed sterilized milkweed leaves until larva showed disinterest in feeding. To ensure that an unforeseen infection by a tainted *A. curassavica* leaf did not occur, each leaf was disinfected with a five percent bleach cleaning solution and rinsed with deionized water. Doses ranged from 2,000, 12,500, and 25,000 spores. The larvae were allowed to continue to grow to pre-pupae. Larvae that failed to consume 80-90% of the milkweed patch were killed and pre-pupae were killed by decapitation, then eviscerated. The digestive tract and other organs were removed. Precautions were taken to not damage the skin tissue.

Histological Preparation

Hypodermal tissue was then transferred to Carnoy's solution. The tissues were dehydrated in a sequence of increasing ethanol concentrations (70%-100%), cleared in xylene, and embedded in paraffin. Table 1 details tissue preparation. Each step immediately after larva hypodermis collection to tissue imbedding in paraffin are listed.

Table 1. List of Times and Reagents

Step	Reagent	Duration (Min)	Step	Reagent	Duration
1	Carnoy's Solution	30	26	Runing Tap H2O	2

Step	Reagent	Duration (Min)	Step	Reagent	Duration
2	70% EtOH	30	27	Ammonia H2O	2
3	80% EtOH	30	28	Running Tap H2O	10
4	90% EtOH	30	29	Eosin	2
5	100% EtOH	30	30	50% EtOH	3
6	100% EtOH	30	31	70% EtOH	3
7	100% EtOH	30	32	95% EtOH	3
8	100% EtOH/Xylene	30	33	100% EtOH	3
9	Xylene	30	34	100% EtOH	3
10	Xylene	30	35	100% EtOH	3
11	Xylene/Paraffin	30	36	100% EtOH/Xylene	3
12	Paraffin	30	37	Xylene	3
13	Paraffin	30	38	Xylene	3
14	Xylene	3	39	Xylene	3

Step	Reagent	Duration (Min)	Step	Reagent	Duration
15	Xylene	3			
16	Xylene	3			
17	100% EtOH	3			
18	95% EtOH	3			
19	80% EtOH	3			
20	70% EtOH	3			
21	50% EtOH	3			
22	DI H2O	5			
23	Harris Hematoxylin	15			
24	Runing Tap H2O	3			
25	1% Acid Destain	6			

Once the tissues were in paraffin, they were situated vertically in a paraffin block and sectioned using the Thermo Scientific Micro HM 340E microtome. Using microscope slides that were fixed with tissue section adhesive solution, tissue sections

were stained as detailed in Table 1. Once completed, the slides were sealed with a layer of resin and covered with glass slips.

To quantitatively assess intracellular tissue infection of *O. elektroscirrha* embedded in monarch hypodermis, hypodermal tissues were examined and measured under light microscopy; measurements were taken with cellSens Standard software (Olympus Corporation, Center Valley, PA, U.S.A). For every monarch, approximately 8 slides with tissue were produced, and two slides were randomly chosen from each group and analyzed for schizont infection. At every pupal dissection, hemolymph smears were taken 8 days post pupation, examined for immune cells, and recorded. All schizonts found in hypodermal tissue were measured (μm) with the area ($A = \pi r^2$) taken for each schizont. An average was taken from each group (ex. Level 2, Infection level 2000) from the calculated areas. To determine if the area taken up in hypodermal tissue by schizont infection was significant either at time of infection or inoculum intensity, an analysis of variance was implemented to see if there was a difference in means with a Tukey's pairwise to locate statistical significance.

Immunological Preparation

Experimental infection protocol for immune cell count was the same as described for histological preparation. Eight days post chrysalis formation, a 5cc 22G$1^{1/2}$ gauge needle was used to puncture the anterior end of the chrysalis, directly opposite of the cremaster. Hemolymph fluid was extracted and deposited onto a slide, then smeared across the length of the slide at a 45-degree angle and allowed to completely dry. The slides were then submerged in laboratory grade methanol for 1 minute, removed, and allowed to dry completely. Circulating hemocytes were smeared and identified by light

microscopy using HEMA 3 stain protocol (Qayum and Telang, 2011). Hemocytes were identified based upon size, morphology, and dye staining properties (Blanco et al., 2017; Hwang et al., 2015; Kwon et al., 2014; Manachini et al., 2011; Qayum and Telang, 2011).

Statistics

To determine the impact of inoculum intensity and time period of infection (instar level) on pathology of instars inoculated, the calculated areas from each tissue section examined are averaged and analyzed through analysis of variance. First, a two-way analysis of variance was conducted to determine if either variable, inoculum intensity or instar level, had effects on one another, in conjunction with the area of intracellular space taken up by schizonts. Then, a one-way analysis of variance was run for each variable, inoculum intensity and instar level on size (area) of schizont measured in hypodermal tissue. These tests will determine whether each variable had a noticeable effect on schizont infection in the tissues. Both two-way and one-way anova were used to see if each variable had an effect on the measured schizont infection in the hypodermis, with the one-way anova measuring variance with just one variable included and the two-way anova measuring both variables effect on infection as well as any combined effect the variables might have. In order to determine if significance occurred between inoculum size, instar level infected, and measured pathological response, a Tukey's multiple comparisons test was employed. To determine at what instar level and inoculum intensity schizont infection was at its highest, parasitic intensity was analyzed, to perform this calculation as well as a one-way analysis of variance and Tukey's multiple comparisons.

To determine if there was a significant increase or decrease in the immunological response of larval hosts, a one-way analysis of variance was run for total immune cell count, individual immune cell type, and parasitic stages found within the smears. For each statistical test run, a Tukey's multiple comparisons test was used to determine if significance occurred for either variable. Data for plasmatocytes, spherulocytes, and prohemocytes had to be log transformed to normalize error variance, although this type of transformation lowers the power of the data set, data must be normalized to prevent skewedness due to non-normal datum measurements. Differences were considered significant when the P-value was less than 0.05 ($*P \leq 0.05$), asterisks denote statistically significant differences between experimentally infected groups. To investigate relationship between spore intensity and overall immunological response a regression analysis was performed.

CHAPTER III

Results

Analysis of *D. plexippus* hypodermal tissue infected with *O. elektroscirrha* schizonts

Following examination of infected host tissue and tissue abstracted from specimens inoculated with uninfected monarch solution (control), 342 schizont parasites were located and measured with the range of area of parasite size 53.18-440.38μm², further described in Table 2. Pictures of micronuclear schizonts in hypodermal tissue with parasitic spores are pictured in Figure 3; schizonts were classified by light microscopy based upon morphology (McLaughlin and Meyers, 1970). To determine if both variables tested, spore inoculum intensity or instar stage of infection, had any combined effect on the amount of intracellular area taken up by schizont infection, the two-way anova was first implemented. From total average intracellular area measured, there were no significant differences when testing spore inoculum intensity with instar stage of infection. However, instar level revealed significant differences ($F_{2,4}$=7.35, df=5, P=0.025), meaning an increase in the intracellular space taken by schizonts in hypodermal tissue, and a Tukey's analysis indicated instar level 5 varying from other groups (see Figure 4). One-way analysis of variance test for spore inoculum intensity versus total average of intracellular space by schizont infection found no variations within means and no significant differences by Tukey's. One-way analysis of variance test for instar level of infection versus average of intracellular space by schizont infection did by variation within means with Tukey's multiple comparisons test finding significant difference within instar level five ($F_{4,7}$=7.72, df=11, P=0.010).

Table 2. Ranges (area) and locations of schizont measurements in monarch larval tissue (μm^2). n = number of specimens.

Instar level	Inoculum Intensity	n	Ranges Measured (μm^2)
2	2,000	28	59.04-380.76
	12,500	29	92.71-297.75
	25,000	27	76.13-199.49
3	2,000	21	84.47-206.34
	12,500	25	81.61-440.38
	25,000	6	131.77-210.03
4	2,000	5	109.07-140.76
	12,500	13	76.47-165.09
	25,000	35	72.78-189.19
5	2,000	36	92.07-325.77
	12,500	44	53.18-340.47
	25,000	73	86.67-304.86

To determine overall infection numbers, individual schizonts were counted across examined tissues. Parasite intensity or total count of schizonts found in hypodermal tissues is graphed in Figure 5, showing a moderate infection in lower level instars and a rise in infection levels in larval stages 4-5. The results seem to indicate spore inoculum intensity size does not affect the parasites ability to infect and occupy tissue in the hypodermis.

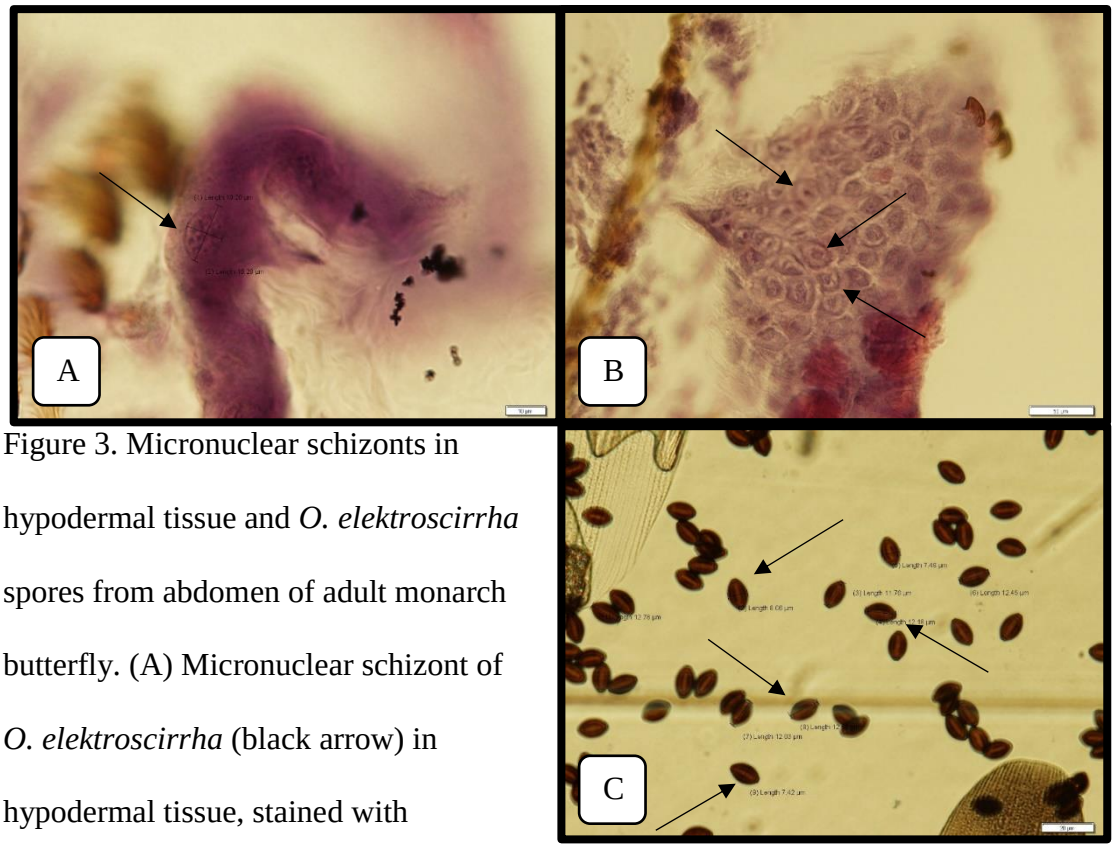

Figure 3. Micronuclear schizonts in hypodermal tissue and *O. elektroscirrha* spores from abdomen of adult monarch butterfly. (A) Micronuclear schizont of *O. elektroscirrha* (black arrow) in hypodermal tissue, stained with hematoxylin-eosin stain. Notice multiple nuclei within cytoplasmic membrane. Bar = 10µm. (B) Micronuclear schizonts (black arrows) collected within a cluster inside the hypodermal layer. Bar = 50µm. (C) Parasitic spores (black arrows) shown in clusters next to monarch scales. Bar = 20µm.

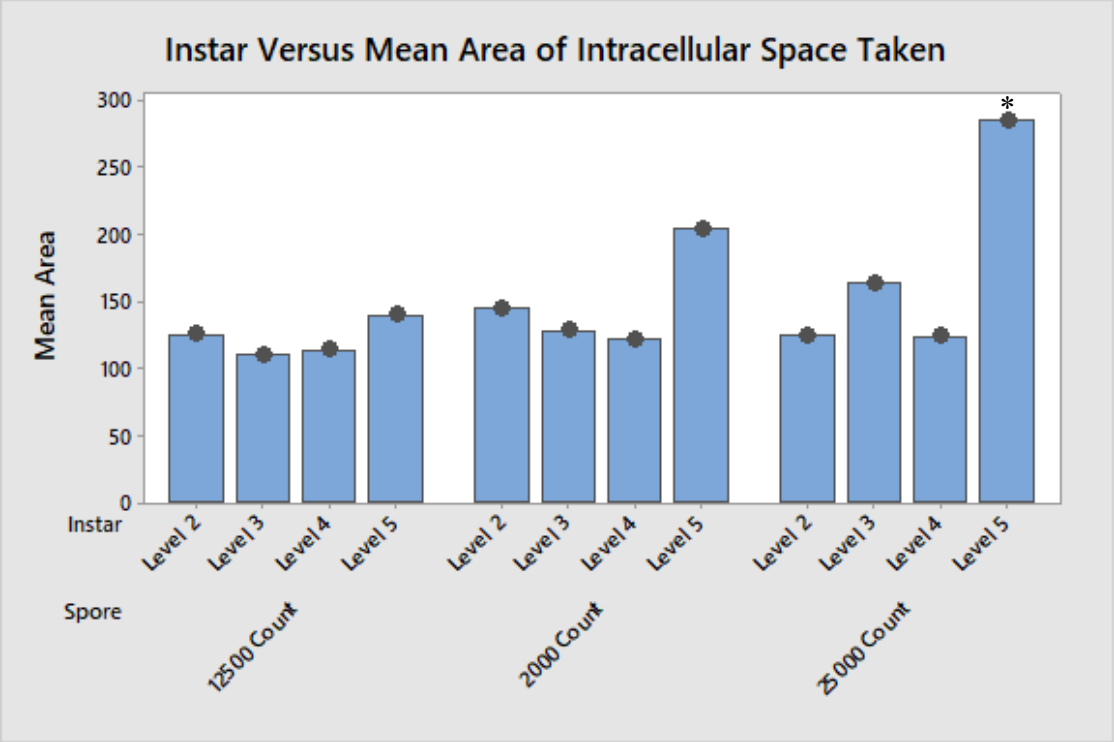

Figure 4. Mean area of Intracellular space taken up by schizont; graphed by level and spore inoculum intensity.

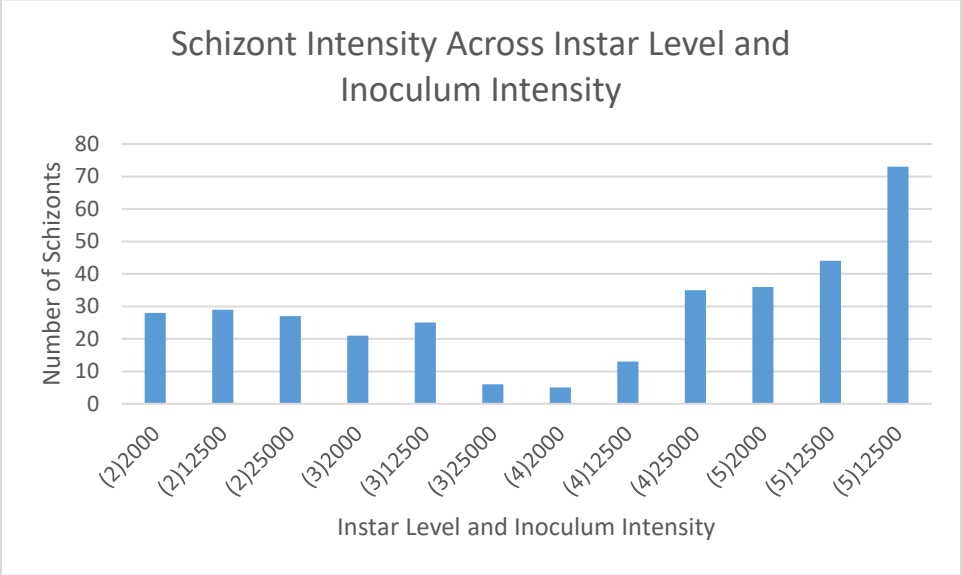

Figure 5. Mean intensity data of schizonts found in hypodermal tissues across instar level and spore inoculum intensity.

Identification and Counting of hemocytes and immune response against *O. elektroscirrha*

Hemocyte types were organized into five morphotypes, including granulocyte (GR), plasmatocyte (PL), oenocytoid (OC), spherulocyte (SP), and prohemocyte (PR). Granulocytes were easily identifiable, and quantitatively were the most numerous cell type found in the smears. Granulocyte cells were approximately 25-35 µm in length and 10-20 µm in width, with several small granules in the cytoplasm and a round nucleus in the center of the cell. Plasmatocytes were elongated and needle shaped in morphology, with cells measuring approximately 20-30 µm in length and 10-15 µm in width. Plasmactocytes were also identified by their 'spindle shaped' nucleus located in the center of the cell body. Oenocytoids were the second most abundant cell type found among all the smears made from hemolymph fluid; these cells were very large and circular shaped. OC cells were approximately 30-40 µm in length and 10-20 µm in width, with a 'moderately sized' nucleus in comparison to the overall size of the cell body. Spherule cells were the least numerous of the cell types identified, with their shape being mostly ovoid or circular, with a small nucleus in the center of the cell body. SP cells were approximately 15-25 µm in length and 5-10 µm in width, and these cells also had small spherules or bubble-like bodies inside the cell space. Prohemocytes were also not very abundant amongst the cell types identified. These cells were distinguished by their large nuclei relative to overall cell size and ovoid shape. PR cells were approximately 20-30 µm in length and 10-15 µm in width.

Statistical analysis of hemocyte types in hemolymph fluid of *D. plexippus*

Statistical analysis of total and individual immune cell type was performed using one-way analysis of variance (ANOVA) with Tukey's pairwise comparison, $P \leq 0.05$ was used to determine statistical significance. A statistical regression was used to determine if a causational relationship between *O. elektroscirrha* infection and immune response of *D. plexippus*. A one-way analysis of variance was used to determine if there was an overall immune response to the infection of *O. elektroscirrha* with varying spore intensity administered at different instar stages showed no statistical significances among treated groups when compared to uninfected controls. To determine if specific immune cell types were present in statistically significantly larger numbers than other immune cell types present in hemolymph fluid, a one-way analysis of variance with a Tukey's Pairwise comparison was conducted on each immune cell type identified within the hemolymph fluid. A log transformation was conducted on the data before analysis of variance tests were implemented for plasmatocytes, spherulocytes, and prohemocytes in order to keep data normalized. Among the five immune cell types analyzed, there were no statistically significant differences when compared to control groups. The regression analysis indicated a low R-value, showing low reliability and no statistically significant causational relationship between spore inoculum intensity administered at specific instar developmental stages and overall immune response.

Identification and Statistical Analysis of *O. elektroscirrha* parasite stages in Hemolymph Fluid and Hypodermal Tissue

Using McLaughlin and Meyers (1970) description of *O. elektroscirrha* parasite stages, an analysis using one-way analysis of variance (ANOVA) and Tukey's pairwise comparison was used to determine statistical significance between number of spores administered at specific instar stages and the number of parasite stages counted in tissue and hemolymph samples post infection. Results indicated there were no statistically significant differences among treatment groups.

CHAPTER IV

Discussion

Intracellular parasites will often infect hosts using a specific mode of transmission. Once inside a cell, they will multiply, burst from that cell with new copies of the parasite, and will continue this stage of the life cycle multiple times, causing damage to the host. This is true for many species in the Phylum Apicomplexa (Kleespies et al., 1997; Xiao and Fayer, 2008; Cowman et al., 2012.)

Ophryocystis elektroscirhha follows a very similar life cycle. Once the spore is ingested and parasites burst from the spores, they will infect surrounding hypodermal cells and multiply, causing damage to surrounding tissues. Which can lead to early death of the host. Based upon the findings of Mcglaughlin and Meyers (1970) parasites were concentrated in the hypodermal section as well as the hemolymph fluid of the chrysalis of pupated monarchs. To include a more comprehensive review of pathological activity by *O. elektroscirrha* infection, this paper details the first inspection of cellular immune reactivity by the host organism.

Link between Pathology and Parasite

The first hypothesis asked if there was a link between the number of spores given to a monarch larva and the number of schizonts. A statistical analysis showed that in this study, this was not the case, as evidenced in Figure 4. Whether the inoculum size was small (2,000) or large (25,000), there was no discernable difference between mean area of schizonts measured in hypodermal tissue and inoculum size given.

There are a few possibilities as to why this result was found: one being that a further analysis of tissues collected may yield more schizonts and show a trend of increasing

parasite numbers. As outlined in similar experimental infection papers, it is possible that the parasites had reached a threshold; too high of an intensity could start to become detrimental to a successful infection within the host, causing a potential decrease in parasite fitness (Benesh and Valtonen, 2007). Researchers have pointed out that in some infections, the age of the parasite has had a noticeable effect on host behavior, a characteristic found in controlled settings, but not often found in the wild. Therefore, this may have been a factor in this experiment, although not likely, as most spores were collected and administered around the same time (Francheschi et al., 2008) As evidenced in Figure 3, parasite mean intensity stayed relatively low despite the initial spore inoculum intensity until the fifth instar, where there was an increase in schizont number in hypodermal tissues. If the reason for significance in the stage five instar was not dependent upon spore intensity size or age, it could be that there is an internal host-hormone versus parasite infection relationship occurring between the two species.

In this study the first hypothesis speculated that with an increase in the number of parasite spores given that there would be more damage done to the larval hosts. However, the only point of statistical significance occurred at a certain age of the host, which with the continued development of young larva there are hormonal, physiological, and morphological changes that occur. It is during the transition into these larva instar stages that hormones are released to drive change in the host, it is possible that during these transitions, there is a relationship between the parasite and host.

In insects, the prothoracic gland produces ecdysone and its subsequent molecule, 20-hydroxyecdysone, as well as several other steroid molecules that are essential to larval growth and development. These include the molting process, metamorphosis, and

maturation of the insect into adult stages in several insects, including those in Lepidoptera (Delbecque et al., 1990; Riddiford and Truman, 1993; R. F. Chapman, 1998; K. Hartfelder, 2000; P. J. Gullan and P. S. Cranston, 2005; Charles A. Triplehorn and Norman F. Johnson, 2005; Yuko S. Niwa and Ryusuke Niwa, 2014 (A); Yuko S. Niwa and Ryusuke Niwa, 2014 (B); Uryu et al., 2015). Another important group of hormones in insects, are juvenile hormones (JH), which have been shown to play a pivotal role in the development of larva, including molting, metamorphosis, and pupation into adult stages (Gilvert et al., 1977; Jones et al., 1982; Lynn M. Riddiford, 1994; Gerard R. Wyatt and Kenneth G. Davey, 1996; R. F. Chapman, 1998; P. J. Gullan and P. S. Cranston, 2005; Charles A. Triplehorn and Norman F. Johnson, 2005; Arnold De Loof, 2006; Fernando G. Noriega, 2014; Yoshiaki Nakagawa and Haruyuki Sonobe, 2016). The concentration of these two groups of hormones that rises and falls in the hemolymph dictates whether or not an immature insect will molt, pupate or develop at all.

Further, internal factors, such as diet and health, as well as external factors, such as environmental cues like temperature and social involvement, play a pivotal role in determining which hormone is needed (H. Frederick Nijhout and Carrol M. Williams, 1974; Riddiford and Truman, 1993; Bloch et al., 1999). It is in the corpora allata that JH hormones are produced and then possibly regulated by several different enzymes, such as JH epoxide hydrolase, which is thought to be produced by fat bodies. JH hormone also has direct and indirect effects on oenocytes as well as prothoracic gland activity, which then would affect ecdysterone production and endocrine levels (Hammock et al., 1975; Lynn M. Riddiford, 1981; Warren et al., 1987; Roe et al., 1993; Stoppie et al., 1998; K. Hartfelder, 2000; Shizuo G. Kamita and Bruce D. Hammock, 2010; Chiang et al., 2016).

It is possible for some neogregarines to parasitize the epidermal layer and oenocytes of insect hosts, and this could have detrimental effects on host health, as there are lipid reserves needed for long-term pupation and the continuation of normal hormone development. There has been some evidence indicating that there is a relationship between parasites and hormone protein synthesis and production, with both being affected by each other's activity (Nancy E. Beckage, 1991; Nancy E. Beckage, 1993; Ana. I. Soldevila and Davy Jones, 1993). It should also be noted that certain macro-nutrients, such as phosphorus, when in limited supply, can affect population dynamics of the host population, and then limit parasitic transmission and exposure, instead of directly affecting parasite growth within the host. This is seen in crustaceans (Pulkkinen et al., 2014), meaning that not only would internal cues such as hormones be affected by parasite growth, but could also be affected by external factors.

It is precisely because of these links between hormone groups like JH Hormone, ecdysteroids and known interactions between hormones in general and parasites that there may be a link between *O. elektroscirrha* and hormonal disruption in the stage five instar, just before pupation. Jones et al., (1982) found two peaks of activity of junior hormone esterase in *D. plexippus*, which is involved in the hydrolyzation of juvenile hormone. The researchers found one peak near the time of wandering, and another during the pre-pupal stage, with both peaks being close to the same level in the hemolymph. Coupling this information with the knowledge that JHE may be produced by fat bodies, it is possible to see *O. elektroscirrha* having a direct effect on this enzyme, and interfering with larval development and maturation, especially since significance was found during

the fifth stage and not during earlier instar stages, when it is thought that the younger instar would be more vulnerable to parasitic infection and pathology.

Specificity of Cell Type for Parasitic Infection

The second hypothesis examined whether multiple cell types would become infected with the parasite. It is typical of neogregarine parasites to be found in the malpighian tubules, oenocytes of the hypodermis, or within the hemolymph (depending on the parasite stage) of its infected host (Mcglaughlin and Meyers, 1970; Kleespies et al., 1997; Valigurova and Koudela, 2006; Yamen and Radek, 2015; 2017). In this study, micronuclear schizonts were found in the hypodermal layer of the monarch larva, however, no parasites were located in oenocytes of the hypodermis. Locations such as the malpighian tubules and other common locations infected by related species of neogregarines found in another insect species were not investigated, except the hemolymph. In the hemolyph of the monarch chrysalis, there were several different stages of the parasite present, including parasitic spores; there was no significant difference, however, between the treatment groups and controls. As McLaughlin and Meyers (1970) hypothesized, the micronuclear schizogony stage is a prolonged and multiplicative stage that does not see much movement of the parasite and is indeed the only stage found in the hypodermal tissues, as was evidenced in the findings of this paper. Producing several nuclei during this stage could be energy intensive for the parasite, perhaps enough to limit movement and confining the parasite intracellularly until the stage five instar undergoes metamorphosis and sexual replication before *O. elektroscirrha* begins to replicate. Although specific tests were not conducted on lipid reserves found through larval tissue, visually there did not seem to be any kind of reduction in overall quantity of lipid

droplets when cells were disrupted. Lipid droplets spilled out from the intracellular compartments of the oenocytes when mechanical destruction of the tissues occurred due to forces of the microtome, however, further testing should be done to rule out this possibility. Analysis of the disruptions in the cuticular layer were not possible due to mechanical destruction of chitinous layers of the larval tissue; no schizonts were found in the chitin region.

Abundance of Parasite found in Hemolymph Fluids

The third hypothesis looked at whether or not there would be an overall increase in the number of individual parasites regardless of stage with an increase in the spore inoculum given, or instar stage at which the larva was infected at. After adding all parasite stages found in hemolymph smears an analysis of variance concluded that there were no significant differences between the number of parasites found and the stage or inoculum size given. There are several different species of intracellular parasites that multiply and have a noticeable increase in the overall abundance after replication, such as those found in genus *Plasmodium.* The expectation with increased inoculum size given to the larva that there would have been a gradual increase in the number of parasites found in the hemolymph smears, seeing as how this was not the case it could be determined that there were mechanisms that prevented an increase in multiplication stemming from a host-parasite interaction, failure to sporulate, or outside environmental factors not accounted for in this study.

Immune Response to Varying Levels of Infection

The final line of analysis looked at whether or not there would be an increase in the immune response in terms of the number of immune cells counted on the hemolymph smears, or more specifically, in certain immune cell types. In this study, there was no significance between the total number of immune cells counted and the instar stage of infection, or spore inoculum intensity given when compared to controls. There was no significance when looking at specific immune cell types when compared to controls. This may be corrected with a large enough sample size to rule out high variability found within these samples. The immunological response measured in this study was count-based, and looked only at the cellular based immune reaction. In order to get a complete understanding of the immune response to parasitic infection, an investigation of the humoral response is needed. Detecting levels of anti-microbial peptides and complimentary proteins could shed light on the total immune response that occurred in these treatment groups.

Parasitism in the Monarch Butterfly

The original question behind this study asked what pathological effects are occurring to monarch larva when infected with the parasite *O. elektroscirrha*. It was hypothesized that with an increase in the number of parasitic spores given to the monarch larva that there would be an increase overall damage and immunological response. The data collected in this study does not support my ideas. With one notable exception it is possible, that the exception seen in stage five larva could have experienced a hormonal change in response to the parasite. This relationship needs further study to elucidate what type of hormonal response is occurring if at all, and how this is affecting the host and parasite.

Instar stage and inoculum size did not seem to increase the size of the measured pathology found in the hypodermal tissues of the infected monarch larva. Although the measured tissue area in the stage five larva was found to be statistically significant, it cannot be held as conclusive evidence that stage five is any predictor of *O. elektroscirrha* infection pathology or survivability. Another avenue for future studies to look at specific areas of interest such as lipid reserves in oenocytes as well as cuticular disruption by *O. elektroscirrha* infection. Although similar studies conducted on the effects of *O. elektroscirrha* infection and subsequent change in monarch fitness point to a detrimental change in metamorphosed hosts, this study found that despite whatever inoculum size was given, there did not seem to be a dramatic detrimental effect on the hypodermal tissue of the monarch larva. It can also be said from this study, that neither larva stage or inoculum size given in the treatments had a noticeable effect on the number of parasite stages found in the hemolymph fluid. This could be a result of failure to sporulate or any other number of outside the scope of this study.

There was no significant difference in the immune response within or among larva stages inoculated with *O. elektroscirrha* or the inoculum size given to the larva. It was thought that with the further development of the larva that the immune response would be more progressive and larger in overall cell count and reflect in the data as a decrease in parasite number in the hemolymph, which was not seen in this study. It was hypothesized that with an increase in the parasite inoculum given to the host that there would be an increase in overall immune cell count, this was not reflected in the data. Possible explanations for this range, one being that the sample size was not large enough to account for the statistical variability seen in the data. Perhaps with a large enough sample size differences

among groups would have arisen and shown a heightened immune response to the infection. It is known that insects lack an adaptive or acquired immune system like what has been documented in mammals and other species. Instead insects solely depend upon an innate immune system consisting of a complex multitude of anti-microbial peptides and proteins as well as a basic assortment of pathogen fighting immune cells for defense. It is in this study that a count of stained immune cells was the basis for quantifying an immunological response in the infected hosts. It is possible that future studies could find a difference in the immune response within or among different stages of infected hosts if examining the anti-microbial peptides and proteins mentioned previously.

Insects play a vital role in the ecosystem as pollinators, decomposers, predators, food source, and that their role is critical to a functioning and healthy ecosystem. It is important to find what role these insects play and to what extent disease afflicts these communities. Several studies have pointed out that change in global temperature, harmful agricultural practices, and spread of disease, have affected the insect community, which has threatened their existence and if action is not taken to protect them their could be negative consequences (Klein et al., 2007; Chagnon et al., 2015; Lindstrom et al., 2016; Sanchez-Bayo and Wyckhuys., 2019). It was based upon the last threat mentioned that this study was conducted. To gain a better understanding of the pathological effects and immune response that occurs in a host, further studies can be conducted to further elucidate the host-parasite dynamics at play. When there is a better understanding of these dynamics, actions and preventative measures can be taken to assist the host (monarch butterflies) in defending itself against harmful opportunistic pathogens and increase the chances for survival and fitness. This study presents that first of its kind in

showing the interplay between the intracellular parasite *O. elektroscirrha* and *D. plexxipus* and that with further studies that perhaps measures can be taken to increase the waning population in the U.S.

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APPENDIX

Approval for image use of Figure 1 by Dr. Jacobus De Roode of Emory University.

RE: Permission Request for Image Use

DJ

De Roode, Jacobus <jderood@emory.edu>
4/8/2019 1:21 PM

To: Mandujano, Christian

Lifecycle.pdf
6.56 MB

Dear Christian,

Yes, please feel free to use. You can credit the image to J.C. de Roode. Attached is a better resolution version.

I'd be interested to hear what you have researched and found out.

Best wishes, Jaap

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PROFESSIONAL SOCIETIES and AFFILIATIONS

- American Society of Parasitologists, member since 2016
- Southwestern Association of Parasitologists, member since 2016

AWARDS and HONORS

- 2016 TPEG Resident Grant

AREAS OF RESEARCH

- Parasitological Pathology
  - Understanding pathology caused by *Ophryocystis elektroscirrha*

CONFERENCES, ABSTRACTS, and INVITED TALKS

Meeting of Southwestern Association of Parasitologists, Kingsville, OK (April 26 – 28, 2019)
Oral Presentation, "Investigating the Effects of Ophryocystis elektroscirrha on the Monarch Butterfly (Danaus plexippus)," Christian A. Mandujano; Tamara J. Cook, Jerry L. Cook, Autumn J. Smith-Herron, Lynne A. M.